

Role of Metabolic Activation in the Carcinogenicity of Estrogens: Studies in an Animal Liver Tumor Model

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Male Syrian golden hamsters chronically exposed to certain synthetic estrogens such as diethylstilbestrol (DES) or 17 α -ethinylestradiol (EE₂) and fed a diet containing 7,8-benzoflavone (BF) develop a high incidence of liver tumors. No such tumors are found in animals treated with estrogen or BF alone. To clarify the role of metabolic activation of the estrogen and BF in the mechanism of hepatocarcinogenesis in this animal model, the effects of pretreatment with DES and EE₂ alone and in combination with BF on the metabolism of DES, EE₂, and BF in hepatic microsomes, isolated hepatocytes, and hamster bile were studied. Hamsters were pretreated for up to 32 weeks. The results clearly show that DES metabolism was not significantly modified under any pretreatment regimen. EE₂ metabolism exhibited a slight increase in 2-hydroxylation after pretreatment with BF and with BF plus EE₂. The most pronounced effect was observed in BF metabolism after pretreatment with BF, with BF plus DES, and with BF plus EE₂: the metabolic rate was increased and several new metabolites that were not found in untreated or estrogen-pretreated animals were formed. These metabolites were tentatively identified as BF-dihydrodiol and dihydroxy-BFs. The formation of these new BF metabolites was accompanied by a change in the activities of certain cytochrome P-450 isoenzymes in hamster liver microsomes. The results of this study imply that metabolic activation of BF rather than of the estrogens plays an important role in the mechanism of carcinogenesis in this animal liver tumor model.

Introduction

Several observations suggest that the formation of reactive metabolites is important in the mechanism of estrogen carcinogenesis (1-3). However, unequivocal evidence is still lacking. A rather convincing argument would be that modulations in the metabolic activation of estrogens were correlated with the ability to induce tumors. A recently described animal tumor model appears to be of particular interest in this context: male Syrian golden hamsters chronically exposed to certain synthetic estrogens such as diethylstilbestrol (DES) or 17 α -ethinylestradiol (EE₂) and fed a diet containing 7,8-benzoflavone (BF) develop a high incidence (80-100%) of hepatic tumors after 6 to 8 months (4). Virtually no liver tumors were observed in animals treated with estrogen or BF alone.

To clarify whether the metabolism of the estrogen and BF is modulated under conditions of tumor induction, we have studied the effects of pretreatment with BF, DES, EE₂, BF plus DES, and BF plus EE₂ on the metabolic pattern of radioactively labeled DES, EE₂, and BF in hamster hepatic microsomal preparations, isolated hepatocytes, and hamster bile *in vivo*. The dosing regimen of pretreatment was the same as used in the carcinogenicity study (4), i.e., DES and EE₂ were implanted SC every 3 months as 20-mg pellets and 30-mg pellets, respectively, and BF was administered in the diet at 0.4%. The effects of the various pretreatments were also measured on the activity of several cytochrome P-450 isoenzymes in hepatic microsomes. The results of these studies, which have been reported in part elsewhere (5-10), are briefly reviewed in this paper.

Results

DES Metabolism

Previous studies in the Syrian hamster *in vivo* (11) and with hepatic and renal hamster microsomes (12) have shown that DES is metabolized to a variety of oxidative products [e.g., 1-hydroxy-DES, 3'-hydroxy-

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DES, Z,Z-dienestrol (Z,Z = DIES), and 1-hydroxy-Z,Z-DIES]. These metabolites can be readily separated and quantified by HPLC (12). When the metabolism of ^{14}C -DES was studied in male hamsters pretreated with DES alone, BF alone, and DES plus BF for 8, 20, and 32 weeks, neither hepatic microsomes (7) nor hepatocytes (9) elicited any alteration in the pattern of DES metabolites as compared to untreated controls. Nonextractable binding to microsomal protein was not increased under any of these conditions. This suggests that the pretreatment regimen leading to tumor formation in the hamster liver is not accompanied by an increased metabolic activation of DES. *In vivo* studies with pretreated hamsters using biliary DES metabolites as probes for hepatic biotransformation support this notion (6).

EE₂ Metabolism

EE₂ can be metabolically activated at the aromatic ring through the formation of the catechol estrogens 2-hydroxy-EE₂ and 4-hydroxy-EE₂ and their further oxidation to the respective semiquinones and quinones (1). In addition, oxidation of the ethinyl group may yield

reactive metabolites. A recent study (13) demonstrated that pretreatment of male Syrian hamsters with BF for 4 months led to a shift in the pattern of microsomal ^{14}C -EE₂ metabolites. A 1.5-fold increase in the formation of 2-hydroxy-EE₂ and a slight increase in the extent of irreversible binding of radioactivity to microsomal protein suggested enhanced metabolic activation of EE₂ after pretreatment with BF. An approximately 2-fold increase in the formation of 2-hydroxy-EE₂ in male hamster liver microsomes was observed in another study after pretreatment for 3 months with EE₂ alone or with EE₂ plus BF (10). However, in the latter study, binding to microsomal protein was found to be decreased about 2-fold in microsomes from all pretreated animals as compared to controls.

BF Metabolism

Hepatic microsomes from untreated male hamsters metabolize ^{14}C -BF to at least five products, as shown by HPLC (Fig. 1). The radioactive peaks were collected and subjected to GC/MS after trimethylsilylation. 6-Hydroxy-BF and 7-hydroxy-BF were unambiguously identified through their mass spectra and co-chroma-

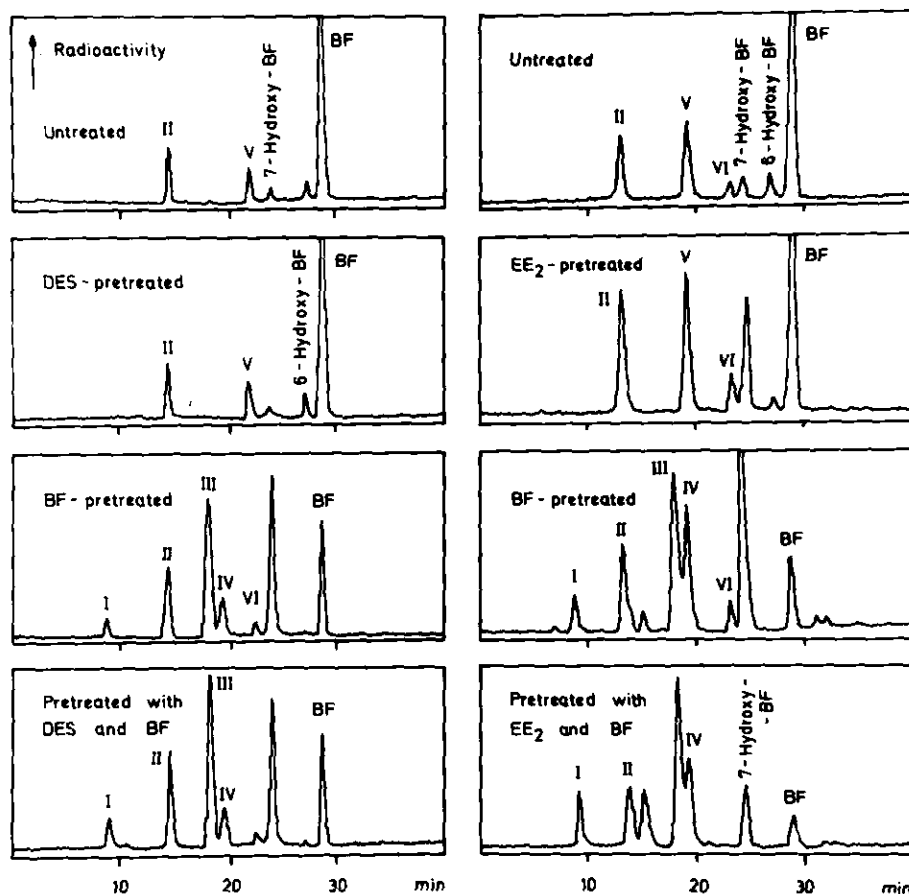


FIGURE 1. Radio-HPLC profiles of BF metabolites generated by hamster liver microsomes after various pretreatments. Left panel: experiments with DES. Right panel: experiments with EE₂. The small differences in retention times between identical samples are due to slight differences in the HPLC conditions. For further details see Blaich et al. (14).

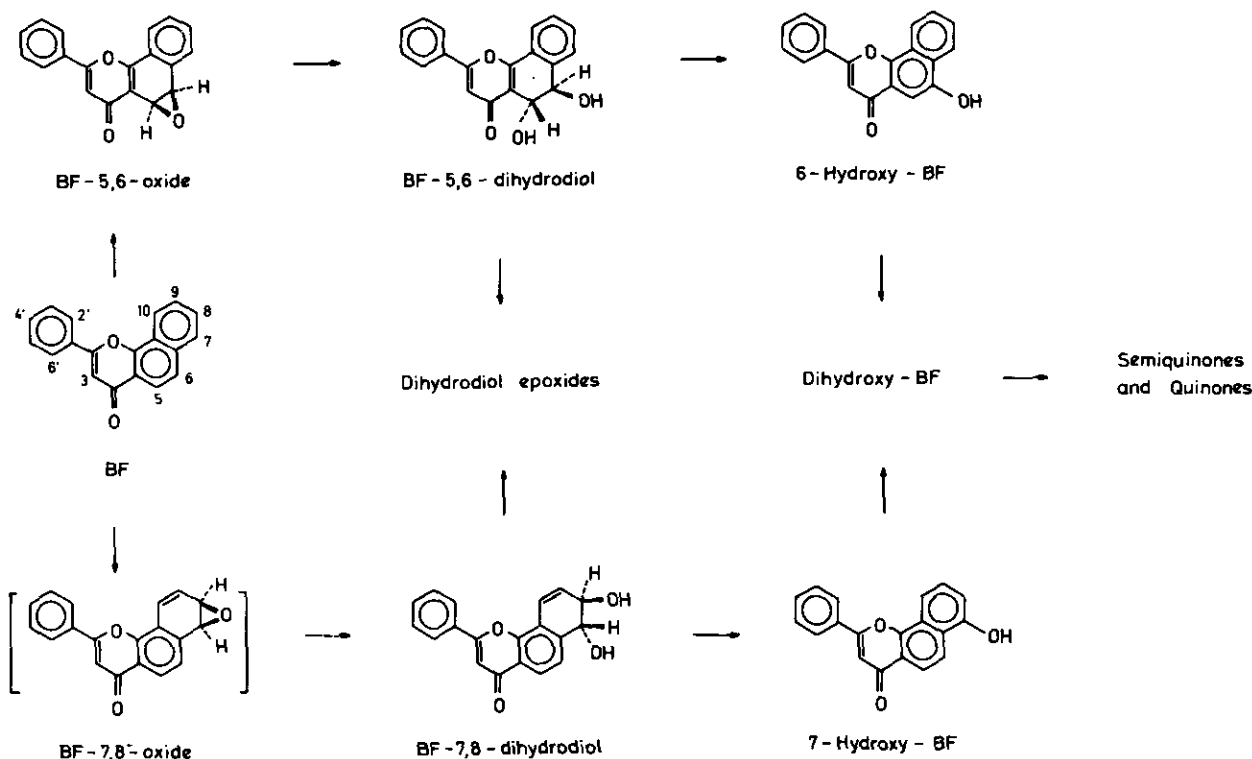


FIGURE 2. Oxidative pathways in BF metabolism possibly leading to reactive intermediates.

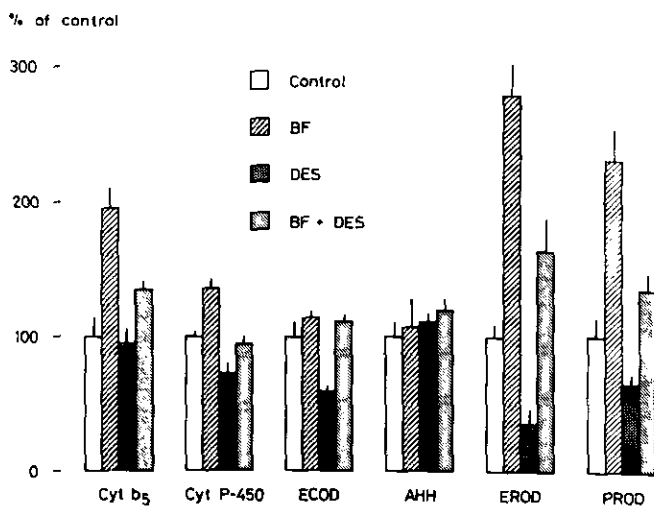


FIGURE 3. Effect of pretreatment (20 weeks) on the levels and activities of various enzymes in hamster liver microsomes (mean \pm SD of five animals). ECOD, 7-ethoxycoumarin-*O*-deethylase; AHH, aromatic hydrocarbon hydroxylase; EROD, 7-ethoxyresorufin-*O*-deethylase; PROD, 7-pentoxoresorufin-*O*-dealkylase.

tography with authentic reference compounds in HPLC and GC. Metabolites II, V, and VI (Fig. 1) were tentatively identified as BF-7,8-dihydrodiol, a dihydroxy-BF, and 4'-hydroxy-BF, respectively. The mass spectra and other evidence for the proposed structures of these products are reported in detail elsewhere (14).

Pretreatment of hamsters with DES or EE₂ did not affect the hepatic microsomal metabolism of BF (Fig. 1). In contrast, pretreatment with BF led to a marked quantitative and qualitative alteration of the metabolic pattern: formation of 7-hydroxy-BF was enhanced, and at least three new metabolites (I, III, and IV, Fig. 1) were observed. Preliminary identification suggests that metabolite I is BF-5,6-dihydrodiol, and metabolites III and IV are dihydroxy-BFs of unknown structure (14). A tentative metabolic scheme of the oxidative metabolism of BF is depicted in Figure 2.

Most interestingly, the pattern of oxidative BF metabolites observed after pretreatment with BF alone was also found in microsomes of hamsters treated with BF plus DES and BF plus EE₂, suggesting that the new metabolites are indeed formed under conditions of liver tumor induction (Fig. 1). Moreover, the effect of pretreatment with BF plus estrogen on BF metabolism was the same in hepatic microsomes and in freshly isolated hepatocytes (9).

Enzyme Activities

The levels of cytochromes P-450 and b₅ and the enzyme activities of 7-ethoxycoumarin-*O*-deethylase (ECOD), aromatic hydrocarbon hydroxylase (AHH), 7-ethoxyresorufin-*O*-deethylase (EROD), and 7-pentoxoresorufin-*O*-dealkylase (PROD) were measured in microsomes from male hamsters after pretreatment with BF alone, DES alone, and BF plus DES for 8 and 20

weeks. The effects were about the same after both periods of pretreatment. As shown in Figure 3, treatment with BF alone increased both cytochrome P-450 and b_5 levels, whereas pretreatment with DES did not affect cytochrome b_5 , but decreased P-450. Combined pretreatment led to an intermediate effect.

After pretreatment with BF, the microsomal activities of EROD and PROD, but not of ECOD and AHH, were significantly induced (Fig. 3). Microsomes from DES-primed animals showed a significant decrease in ECOD, EROD, and PROD activities; AHH activity was not affected. The combined pretreatment with DES and BF had different effects on the activities of the different enzymes. ECOD and AHH showed no significant alteration; EROD and PROD were clearly induced, although to a lesser extent than in animals pretreated with BF alone.

Discussion

The induction of hepatocellular carcinomas in the male Syrian golden hamster by simultaneous treatment with estrogen and BF has been proposed as an animal model for liver tumors in women taking oral contraceptives (4). The mechanism of tumor formation in this animal model is as yet unclear.

Our data indicate that pretreatment of hamsters with BF results in a marked increase in the level of total cytochrome P-450 and in the activities of various P-450 isoenzymes in the liver. Although BF acts as an inducer, the metabolism of DES remains unaffected. This implies that the P-450 isoenzymes inducible by BF are not primarily involved in DES metabolism.

A somewhat different situation appears to exist for EE_2 , because BF induction leads to an increase in 2-hydroxylation, a pathway possibly involved in the metabolic activation of steroidal estrogens. However, it is unlikely that the observed change in EE_2 metabolism is the sole reason for the high incidence of hepatocellular carcinomas observed after combined treatment with EE_2 and BF because the same pathway is stimulated after pretreatment with EE_2 alone, which gives rise to only a very low incidence of liver tumors (4). Thus, it must be concluded that other factors are involved in hepatocarcinogenesis in this animal model.

The striking alteration of BF metabolism observed under the conditions of hepatic tumor formation suggest that reactive metabolites of BF may play an important role. According to the preliminary identification, the altered metabolism yields dihydrodiols and dihydroxylated BFs that may give rise to reactive electrophiles such as dihydrodiol epoxides and semiquinones/quinones.

It is interesting in this context that rat liver microsomes induced by 2,3,7,8-tetrachlorodibenzodioxin (TCDD) yield similar BF metabolites, which have been demonstrated to be clastogenic and to produce DNA adducts in Chinese hamster ovary cells (15,16). As no hepatic tumors were obtained with BF alone (4), it is

likely that BF acts as a pure initiator in the hamster liver, and the promoting activity of DES is required for tumor manifestation. A similar situation appears to prevail for the treatment with EE_2 and BF, with the exception that EE_2 may be a weak initiator in its own right, as discussed above, in addition to acting as a promotor. In accordance with this hypothesis is the observation that treatment with EE_2 plus BF leads to a higher tumor incidence and a shorter latency period than treatment with DES plus BF (4).

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